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Chromatin immunoprecipitation reveals a novel role for the *Drosophila* SoxNeuro transcription factor in axonal patterning

Franck Girard *, Willy Joly, Jean Savare, Nathalie Bonneaud, Conchita Ferraz, Florence Maschat *

Institut de Génétique Humaine, Centre National de la Recherche Scientifique UPR1142, 141 rue de la Cardonille, 34396 Montpellier Cedex 5, France

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Abstract

In all metazoans, the expression of group B HMG domain Sox transcription factors is associated with the earliest stages of CNS development. In *Drosophila*, *SoxNeuro* (*SoxN*) is involved in dorso-ventral patterning of the neuroectoderm, and in the formation and segregation of neuroblasts. In this report, we show that *SoxN* expression persists in a subset of neurons and glial cells of the ventral nerve cord at embryonic stages 15/16. In an attempt to address *SoxN* function in late stages of CNS development, we have used a chromatin immunoprecipitation approach to isolate genomic regions bound in vivo by SoxN. We identified several genes involved in the regulation of axon scaffolding as potential direct target genes of SoxN, including *beat1a*, *semaphorin2a*, *fasciclin2*, *longitudinal lacking* and *tailup/islet*. We present genetic evidence for a direct involvement of *SoxN* in axonal patterning. Indeed, overexpressing a transcriptionally hyperactive mutated SoxN protein in neurons results in specific defects in axon scaffolding, which are also observed in transheterozygous combinations of *SoxN* null mutation and mutations in its target genes.

Keywords: SoxNeuro; Sox; HMG; *Drosophila*; Axon guidance; Transcriptional control

Introduction

It is now well established that some of the early events of central nervous system (CNS) development have been conserved during metazoan evolution (Sasai, 2001). In both vertebrates and *Drosophila*, the antagonistic activities of secreted proteins, BMP4/chordin in vertebrates, and their homologues Dpp/Sog in fly, result in the subdivision of the neurogenic versus non-neurogenic regions of the ectoderm. Among the targets of chordin/Sog action are the Sox genes, encoding HMG domain transcription factors conserved during evolution (Mizuseki et al., 1998; Cremazy et al., 2000). Genes of the Sox family are involved during metazoan development in regulating multiple events of cell fate determination and cell proliferation/differentiation in a number of tissues, including CNS, gut, testis, bones or eyes (Wegner, 1999; Kamachi et al., 2000; Savare and Girard, 2002; Wilson and Koopman, 2002; Kondoh et al., 2004; Pevny and Placzek, 2005). Early neurogenesis in vertebrates and invertebrates is associated with the expression of group B Sox genes (see Savare and Girard, 2002 and references therein). In *Drosophila*, the group B Sox genes, *SoxNeuro* (*SoxN*) and *Dichaete* are required at early steps of neurogenesis, in the dorso-ventral patterning of the neuroectoderm and in the formation of neuroblasts (NBs) (Buescher et al., 2002; Overton et al., 2002; Zhao and Skeath, 2002). Indeed, in *SoxN* null mutant, neuroectodermal cells fail to be singled out as NBs, and delamination does not take place. Since the expression of the proneural genes of the Achaete-Scute complex (AS-C) is partially, but not completely, lost in *SoxN* and *Dichaete* mutants, it was suggested that these Sox genes act both upstream of, and in parallel to, the proneural AS-C genes in the neuralizing pathway, likely in combination with the columnar homeodomain genes *vnd*/*ind*/*msh* (Buescher et al., 2002; Overton et al., 2002; Zhao and Skeath, 2002; Gomez-Skarmeta et al., 2003).

Although the function of Sox genes during CNS development starts to be elucidated, less is known on the target genes they regulate. In the case of group B Sox proteins, only few

* Corresponding authors. Fax: +33 4 99 61 99 57.
E-mail addresses: fgirard@igh.cnrs.fr (F. Girard), florence.maschat@igh.cnrs.fr (F. Maschat).

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target genes have been identified, including in mammals *nestin* (Tanaka et al., 2004), *fgf4* (Ambrosetti et al., 1997), *HoxB1* (Di Rocco et al., 2001), *UTF1* (Nishimoto et al., 1999), and *crystallin* (Kamachi et al., 1995), and in *Drosophila crystallin* (Blanco et al., 2005), *slit* (Ma et al., 1998) and *vnd* (Yu et al., 2005). Several methods have been developed in the past few years towards a genome wide identification of target genes regulated by a given transcription factor, including DNA microarrays, chromatin immunoprecipitation (ChIP), and ChIP on chip studies combining both techniques, which was essentially used in yeast (reviewed in Hanlon and Lieb, 2004). *SoxN* is also expressed later during embryogenesis in the ventral nerve cord (VNC) and in the brain (Cremazy et al., 2000, 2001; Buescher et al., 2002), suggesting a role in the CNS late in embryogenesis. In an attempt to decipher the events downstream of *SoxN*, we have used a chromatin immunoprecipitation procedure towards a genome wide identification of genomic DNA regions bound in vivo by SoxN. This strategy led to the identification of several genes that constitute potential direct targets of SoxN transcriptional activity, a number of them being involved in axon guidance/pathfinding. We show that *SoxN* is expressed in a subset of neurons and glia within the embryonic VNC. We also provide genetic evidence that *SoxN* is involved in axonal patterning, likely through the direct regulation of these genes in the embryonic VNC.

Material and methods

Fly strains

All flies were raised on standard medium at 25°C. OregonR was used as wild type. The following strains were used: UAS-SoxNK439R (Savare et al., 2005); $SoxN^{GA1192}$ (kindly provided by W. Chia); *elav*-GAL4, tup^{1}, $beat1a^{3}Fas3^{E25}$, $lola^{00642}$, $Sema2a^{03021}$ and Ubx^{101} (The Bloomington Stock Centre), *islet*-tau-myc-GFP (kindly provided by S. Thor), *eagle*-Gal4 (kindly provided by A. Garces), UAS-mCD8-GFP. GFP marked balancer chromosomes were used to unambiguously identify mutant embryos.

Chromatin immunoprecipitation from Drosophila embryonic nuclei

Chromatin immunoprecipitation was performed essentially as already described (Solano et al., 2003). Nuclei from OregonR embryo collection enriched in late stages (stages 11 to 16) were prepared through sucrose gradient centrifugation, then UV-irradiated for 4×2.5 min at 2.400 Joules using Stratalinker. Nuclei were lysed, and the chromatin was prepared by centrifugation in CsCl containing buffer. Chromatin was sonicated, in order to obtain genomic DNA fragments ranging in size from 200 bp to 3 kbp. For immunoprecipitation, chromatin was first preincubated with protein A–agarose (Sigma), for 4 h at 4°C, in buffer 1 (20 mM Tris–Cl pH 7.5, 100 mM Nacl, 1 mM EDTA, 0.1% BSA). Pre-cleared chromatin was then incubated with either preimmune serum or with rabbit anti-SoxN serum, and protein A–agarose beads overnight at 4°C. Beads were washed 3 times in buffer 2 (10 mM Tris–Cl pH 7.5, 0.5 M LiCl, 0.1% SDS, 2% NP40), and 3 times in buffer 3 (50 mM Tris–Cl pH 7.5, 50 mM NaCl). Bound chromatin was eluted from the beads with 1.5 volume of buffer 4 (10 mM Tris–Cl pH7.5, 100 mM NaCl, 1 mM EDTA, 4% Sarcosyl, 2 mM AEBSF), for 30 min at 37°C, recovered by centrifugation and dialyzed overnight at 4°C in buffer 5 (Tris 10 mM pH 7.5, EDTA 1 mM). Immunoprecipitated chromatin was successively incubated with Klenow (30 min at room temperature), RNase A (50 μg/ml for 30 min at 37°C), and proteinase K (0.5 mg/ml for 3 h at 65°C, in the presence of 1% SDS). DNA was phenol/chloroform extracted, and ethanol precipitated in the presence of glycogen. Linkers were prepared by mixing an equal volume of 5 μM solutions of oligo 20 mer (5′ctgctcgaattcaagctt) and oligo 24 mer (5′P agaagcttgaattcgagcagtcag). Mixed oligos were annealed 3 min at 100°C, and renatured at room temperature for 60 min. Precipitated chromatin was recovered in 14 μl H_2O, and ligated with linker (50 nM final) and T4 DNA ligase. The ligation reaction was directly used for PCR amplification in the presence of oligo 20 mer (1 μM final). The PCR amplified material was cloned in HindIII digested pBlueScript. Individual clones from the SoxN immunoprecipitated DNA were sequenced. For in silico analysis, the position of the immunoprecipitated fragments was determined by Blast using Release 4.1 of the *Drosophila melanogaster* genome.

Southern blot

DNA clones corresponding to intergenic and intronic fragments (see Result section for details) were HindIII digested and run on 2% agarose gels. DNA was transferred to nitrocellulose membranes and processed for hybridization using standard procedure. Membranes were first hybridized with ^{32}P labeled (Rediprime kit, Amersham) control library, and autoradiographed. After dehybridization, membranes were hybridized with ^{32}P labeled SoxN library and autoradiographed (as described in Solano et al., 2003). The two signals, SoxN versus control, were compared for each of the clones tested.

Electrophoretic mobility shift assay

EMSA were performed essentially as described previously (Solano et al., 2003). As protein source, we used purified GST and GST-SoxN (Bonneaud et al., 2003). For super shift, rabbit polyclonal SoxN antibody was used (Cremazy et al., 2001). Plasmids containing multimerized Sox binding sites (Bonneaud et al., 2003) were used for control and competition experiments.

Embryo immunostaining and in situ hybridization

Whole-mount embryo immunostaining and in situ hybridization experiments were performed using standard procedures (Tautz and Pfeifle, 1989). The following primary antibodies were used: Cy3 conjugated mouse anti-HRP (Jackson Immunoresearch), affinity purified rabbit anti-SoxN (Cremazy et al., 2001), polyclonal rat anti-SoxN (Cremazy et al., 2000), mouse and rabbit anti-GFP (Molecular probes), mouse anti-myc 9E10 (Santa Cruz), monoclonal anti-repo, anti-Elav, anti-eve, anti-en (Developmental Studies Hybridoma Bank), monoclonal anti-Ubx (a gift of J.M. Dura), polyclonal serum against Dichaete (a gift of J. Nambu). Secondary antibodies used were biotinylated anti-rabbit (Amersham) detected with avidin HRP (Vectastain), Alexa conjugated anti-mouse and anti-rabbit (Molecular probes) and Cy2 or Cy3 conjugated anti-mouse, anti-rat and anti-rabbit (Jackson Immunoresearch). Fillet preparations of stages 15/16 embryos and subsequent immunostainings were performed as described (Benveniste et al., 1998). *Beat1a* cDNA (kindly provided by C. Goodman) was used to generate DIG labeled antisense RNA probe according to manufacturer instruction (Roche).

Results

Isolation of genomic DNA fragments bound in vivo by SoxNeuro

We have previously reported a ChIP-based approach to isolate at a large scale *Drosophila* genomic DNA fragments bound in vivo by the homeodomain transcription factor Engrailed, which led to the identification of numerous Engrailed direct target genes (Solano et al., 2003). ChIP is now routinely used for the analysis of single, well-defined promoters, or at the genome level in yeast, but only very few studies have reported the use of this technique towards a genome wide identification of target genes of transcription factors in animals. Reports where the immunoprecipitated DNA were cloned and sequenced include the studies on Engrailed (Solano et al., 2003) and

kruppel (Matyash et al., 2004) in *Drosophila*, STAT5 (Nelson et al., 2004), BARX2 (Stevens et al., 2004) and E2F (Weinmann et al., 2001) in humans. Thus, ChIP emerged very recently as a possible reliable technique towards a genome wide identification of direct target genes of transcription factors. In addition, recent ChIP on chip studies, combining ChIP and DNA microarrays, have been employed to analyze binding sites for several transcription factors in humans, but these studies are still limited by the availability of microarrays covering large genomic regions (reviewed in Sikder and Kodadek, 2005).

The ChIP approach was applied here to the SoxN transcription factor. As starting biological material, we used a wild-type embryo collection enriched in late stages (stages 11 to 16), aiming to isolate genes regulated by SoxN during neuronal differentiation and axonal scaffolding. Briefly, the protocol consisted in several steps: freezing of DNA/protein interactions through UV treatment, preparation of chromatin, immunoprecipitation with anti-SoxN antibody, elution of co-immunoprecipitated DNA, PCR amplification, cloning and sequencing of immunoprecipitated DNA fragments (detailed in Material and methods). Two DNA libraries were prepared: one obtained with anti-SoxN antibody (referred to as SoxN library), and one with preimmune serum (referred to as Control library) (Fig. 1A). 249 clones were sequenced from the SoxN library, and were mapped in the *Drosophila* genome to 125 distinct genomic positions. Of these fragments, 36 (28.8%) corresponded to intergenic regions, 28 (22.4%) to introns, 33 (26.4%) to exons and 28 (22.4%) to repeat DNA. For our analysis, we considered only intergenic and intronic fragments (64 fragments). To determine whether the ChIP experiment gave significant targets above random background, we verified how many of these fragments were indeed enriched through the anti-SoxN immunoprecipitation. We performed Southern blot analysis on these 64 clones, probing with either ^{32}P labeled SoxN library or control library. We considered that enrichment was significant when the ratio SoxN/control was higher than 2, which appeared to be the case for 25 clones out of the 64 tested by Southern (shown in Fig. 1B is the result of the Southern experiment for 12 clones). These 25 clones correspond to 26 genomic positions, 17 intronic and 9 intergenic. Indeed, one chimera clone contained two fragments hitting the genome at two distinct positions (Table 1, and on line Supplemental data for the sequence and position of the clones). The length of the recovered fragments varies from 44 bp to 359 bp. It is important to note that 11 clones out of these 25 contain an endogenous HindIII restriction site at one (or both) extremity. The linker used to PCR amplify the fragments contained an HindIII site, and this enzyme was used to digest the PCR amplified DNA for cloning into pBlueScript. Thus, for these 11 clones, the initial immunoprecipitated fragments were longer, and a shorter DNA was cloned after HindIII restriction. We found that a majority of these fragments (17/25) are located within introns, thus in regions outside of, and sometimes far away from, gene promoters. This observation was already made by us (Solano et al., 2003) and others (Matyash et al., 2004; Stevens et al., 2004), and suggests that these regulatory regions might function through long-range interactions perhaps involving chromatin looping. In addition, although only intronic and intergenic regions were analyzed here, it remains possible that sites mapping to exons (see on line Supplemental data for sequence) would also be candidates for potential regulatory activities. This is particularly interesting in the case of SoxN,

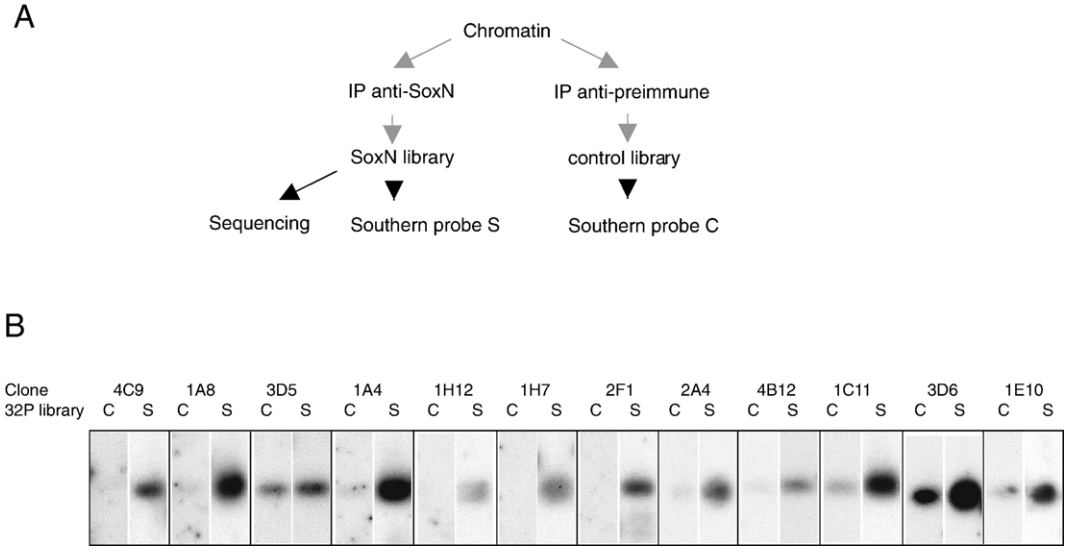

Fig. 1. Southern blot hybridization test on 12 clones. (A) Schematic representation of the ChIP experiment and control. Two immunoprecipitations were performed from chromatin prepared from embryonic nuclei, one with the anti-SoxN antibody, and one with preimmune serum, which led to two DNA libraries, SoxN (S) and control (C). Only the individual clones from the SoxN library were sequenced. These two libraries were also ^{32}P labeled, and used in Southern experiment to probe 64 clones from the SoxN library. (B) The two ^{32}P labeled libraries used were C: control and S: SoxN. A total of 64 clones from the SoxN library were analyzed by Southern, and 25 were shown to be significantly enriched through ChIP (when the ratio signal obtained with the S probe/signal obtained with the C probe was over 2). The clones shown here correspond to the following genes (see also Table 1): *trol/CG13758* (4C9), *Fas2* (1A8), *CG1631/CG1504* (3D5), *PLC21C* (1A4), *Ubx* (1H12), *beat1a* (1H7), *DNCad2* (2F1), *Rs1* (2A4), *CG12911* (4B12), *rexin* (1C11), *Gsα60A* (3D6), *bnl* (1E10).

Table 1
Listing of SoxNeuro target genes

Cytology	Candidate gene(s)	Gene ontology	Length (bp)
Immunoprecipitated fragments in intronic regions			
4B1–3	*Fasciclin 2* (a) (*Fas2*)	Neural cell adhesion; axon pathfinding	49
21B8-C1	*PLC21C*	Enzyme (phospholipase C), GPCR signaling pathway	44
35E2	*Beat1a*	Neural cell adhesion; axon pathfinding	109
36D3	*Cadherin N2*	Neural cell adhesion? axon pathfinding?	57
44B7	*Rs1*	Enzyme (RNA helicase)	170
46F9	CG12911	Unknown	130
46F9–47A1	*Rexin/CAP*	Vinculin binding; *expressed in embryonic sensory nervous system*	215
47A11–47A13	*Longitudinals lacking* (*lola*)	Transcription factor; axon pathfinding	138
53C6–7	*Semaphorin2a* (*sema2a*)	Neural cell adhesion; axon pathfinding	199
55B12	CG5719	Enzyme (guanylate cyclase), GPCR signaling pathway	170
57B9–12	CG30296	Unknown	359
60A12–13	*Gsα60a*	G protein; GPCR signaling pathway; learning and memory	58
66D12–15	CG32030	Actin binding; *expressed in midline glia*	168
89D6–9	*Ultrabithorax* (*Ubx*)	Transcription factor; NB/neuron segmental identity	146
92B2–3	*Branchless* (*bnl*)	FGF receptor	205
96A7	CG6643 (a)	Unknown	248
100A6	CG12045	Unknown	162
Immunoprecipitated fragments in intergenic regions			
3A4–6	CG13758	Neuropeptide receptor, GPCR signaling pathway	338
	Trol/perlecan	Heparan sulfate proteoglycan; larval NB division	
13B6	CG15032 (a)	Unknown	63
	gce	Transcription factor	
19C4	CG1631	Unknown	62
	CG1504	Receptor	
34C6	*Rsu-1*	Small GTPase interacting protein	135
	CG18507	Unknown	
37B5	*Tailup/islet* (*tup*)	Transcription factor; neuronal fate specification; axon pathfinding	60
	CG18397	Unknown	
62A12	CG12011	Unknown	391
	CG13930	Enzyme (dynein ATPase)	
68A4	*Mocs1*	Molybdopterin cofactor synthesis	44
	CG6310	Unknown	
91B3–4	CG7691 (a)	Nucleic acid binding	347
	Fruitless (*fru*)	Transcription factor; neuronal fate specification; axon pathfinding	
92F8–10	CG31205	Enzyme (protease)	182
	Oamb	Amine receptor; learning and memory	

Given are the cytological position, as described in Flybase (http://flybase.bio.indiana.edu/), the length of the fragments, the candidate target gene(s), their molecular function according to Gene Ontology, and when available their involvement in CNS development. For genes of unknown function, CNS expression is given when available (data extracted from Flybase). (a) Corresponds to chimeric fragments, containing two fragments mapping at different genomic positions, that are likely to result from the cloning procedure. GPCR: G-protein-coupled receptor. NB: neuroblast. See also Supplemental data available on line, containing the sequence of the immunoprecipitated clones, their position within each genes, and the presence of Sox consensus binding sites.

since Sox proteins are known to induce significant DNA bending, which could be important in the formation of higher order chromatin structures (Kamachi et al., 2000; Wilson and Koopman, 2002).

Identification of SoxNeuro target genes

To assign for potential SoxN target genes, we considered a single target gene when the fragment was located within an intron of this gene, and the two surrounding genes in the case of intergenic fragments, regardless the distance to the transcription start. The listing of potential SoxN target genes is given in Table 1. These genes can be categorized into several classes, encoding transcription factors (*lola*, *Ubx*, *fruitless*, *gce*, *tailup/islet*), enzymes (*PLC21C*, *Rs1*), receptors and associated proteins (*Gsα60A*, *branchless*, *Oamb*), neural cell adhesion molecules (*fas2*, *beat1a*, *DNCad2*, *sema2a*, *trol*). In the case of intronic fragments, it is interesting to note that among the known candidate target genes, most of them (8/11) are involved, or at least specifically expressed, in late steps of CNS development. Several of the isolated SoxN target genes are involved in axonal patterning, a process in which axons are guided along specific pathways by attractive and repulsive cues in the extracellular environment (reviewed in Cooper, 2002; Dickson, 2002; Huber et al., 2003). Among these, *beat1a* encodes a secreted protein of the immunoglobin superfamily, which functions as an anti-adhesive factor secreted by motor neuron growth cones at defasciculation choice points (Fambrough and Goodman, 1996). *Fas2* encodes a protein of the immunoglobin superfamily expressed in longitudinal axons, which displays adhesive function in axon fasciculation (Grenningloh et al., 1991) and in growth cone guidance (Lin

et al., 1994). *DNCad2* encodes a cell adhesion molecule closely related to CadN. Although *DNCad2* function in neural cell adhesion has not been examined, one can speculate a function in axon patterning like *CadN* (Iwai et al., 1997). In support of this, we found that *DNCad2* expression was restricted to the CNS at embryonic stage 16 (data not shown). *Sema2a* encodes a transmembrane protein expressed in a subset of VNC neurons, exhibiting a chemorepulsive activity that deflects axons away from inappropriate regions (Huber et al., 2003). *Lola* encodes a BTB-POZ transcription factor involved in establishing the pattern of longitudinal axon projections, both by inhibiting growth across the midline and promoting axon growth longitudinally, likely through the regulation of multiple independent guidance genes (Crowner et al., 2002). *Tup* (also named *Islet*) encodes a LIM-homeobox transcription factor expressed in interneurons and in post mitotic motor neurons projecting to ventral targets, and involved in motor neuron identity (Thor and Thomas, 1997) and axon pathfinding through the regulation of guidance genes such as *beat1c* (Certel and Thor, 2004). *Ubx* encodes an homeobox transcription factor involved in NB segmental identity (reviewed in Doe and Scott, 1988), that also appears to be important for axon patterning in the VNC (Merritt and Whitington, 2002). *Fruitless* encodes a BTB-POZ transcription factor involved in multiple developmental processes, including axon fasciculation in the embryonic CNS (Song et al., 2002). Therefore, these genes affect axon guidance/pathfinding at different levels, transcriptional or extracellular, acting either as repellent or attractive cues.

In vitro binding of SoxNeuro to immunoprecipitated DNA fragments

In vitro, vertebrate Sox proteins bind DNA to the degenerated consensus sequence $^{A}/_{T}^{A}/_{T}CAA^{A}/_{T}$ (Pevny and Lovell-Badge, 1997). When examining Sox binding sites in regulatory sequences of known SoxB target genes, it appears that all fit to this consensus, with sometimes a degree of divergence in the nucleotides flanking the ACAA core (Kamachi et al., 1995; Ambrosetti et al., 1997; Nishimoto et al., 1999; Di Rocco et al., 2001; Tanaka et al., 2004; Blanco et al., 2005). *Drosophila* Dichaete also binds this type of consensus in vitro (Ma et al., 1998). SoxN efficiently binds this consensus in vitro, as revealed by electrophoretic mobility shift assays (EMSA) using 7SOX probe, containing seven AACAAAG sites (Fig. 2A and Bonneaud et al., 2003). Competition with increasing quantities of cold 7SOX probe, as well as incubation with anti-SoxN antibody confirmed the specificity of the binding (Fig. 2A). We next analyzed by EMSA the ability of SoxN to bind the immunoprecipitated DNA fragments. SoxN binds the 1H12 fragment, localized in an *Ubx* intron (Fig. 2B). Again, the specificity of the binding was demonstrated with incubation with cold 7SOX probe, which competed for SoxN binding to 1H12, and with incubation with SoxN antibody, which induced super shift of the 1H12-SoxN complexes. In both cases, GST protein alone did not form complex with the radiolabeled probe (not shown). Similar observations were made for the following fragments: 4A7 (localized between *tup* and *CG18397*) and 1H7 (localized in *beat1a* intron). In these two cases, specific complexes were observed with GST-SoxN protein and not with GST alone, and the formation of these complexes was competed, completely or partially, with the cold 7SOX probe (Figs. 2C, D). Together, these data demonstrate that SoxN can specifically bind in vitro to several of the DNA fragments isolated by ChIP. This binding is likely to involve the $^{A}/_{T}^{A}/_{T}CAA^{A}/_{T}$ consensus, since it is present in most of the immunoprecipitated fragments (see Supplemental data available on line). Indeed, 9 out of 17 intronic fragments, and 8 out of 9 intergenic fragments, contain one or more typical Sox consensus binding sites. This is for example the case for the 1H12 and 4H7 fragments, containing respectively two and one Sox binding sites. As mentioned before, a certain degree of divergence is observed in the nucleotides flanking the ACAA core. It is noteworthy that of the 8 intronic fragments that do not contain the typical Sox consensus binding site, 6 contains the ACAA core, among which is the 1H7 fragment (see Supplemental data available on line).

SoxNeuro is expressed in a subset of neurons and glia in the ventral nerve cord

SoxN has been implicated in the formation of NBs, upstream of the Achaete-Scute complex genes (Buescher et al., 2002; Overton et al., 2002). *SoxN* is expressed early during embryogenesis in the neuroectoderm and in forming NBs, and later in the VNC and the brain (Cremazy et al., 2000, 2001; Buescher et al., 2002). SoxN immunostaining in late embryonic stages revealed a reiterated pattern in a subset of cells within the VNC, around 25–30 per abdominal segment (Figs. 3A–C). For a closer examination of *SoxN* expression, immunostainings were performed on dissected VNC from embryos at stages 15/16. Double immunostaining with the neuronal specific marker Elav showed that several of the SoxN-positive cells are neurons (Figs. 3D–F). Double immunostaining with the glia specific marker Repo (Halter et al., 1997) revealed that SoxN is expressed in several glial cells, 6–7 per abdominal hemisegment (Figs. 3G–J). Based on their shape and location (Ito et al., 1995), the medial most cells might correspond to MM-CBG (medial most cell body glia, two in each thoracic hemineuromere and one in abdominal hemineuromere), while the more laterally located might correspond to SPG (subperineurial glia). Together, these experiments demonstrate that *SoxN* is expressed in a restricted population of neurons and glia within the VNC. This suggests that in addition to its early function within the neuroectoderm, SoxN might also be required at late steps of embryonic neurogenesis, in agreement with the finding that axon patterning genes are potential SoxN target genes.

The nature of SoxN expressing neurons was investigated in immunostaining experiments with several neuron specific markers. At stage 16, the expression in the VNC of the Sox gene *Dichaete* is restricted to two clusters of cells in each of the thoracic segments and a single cell in each of the abdominal segments (Soriano and Russell, 1998 and Fig. 3K). Since *Dichaete* and *SoxN* were shown to function synergistically

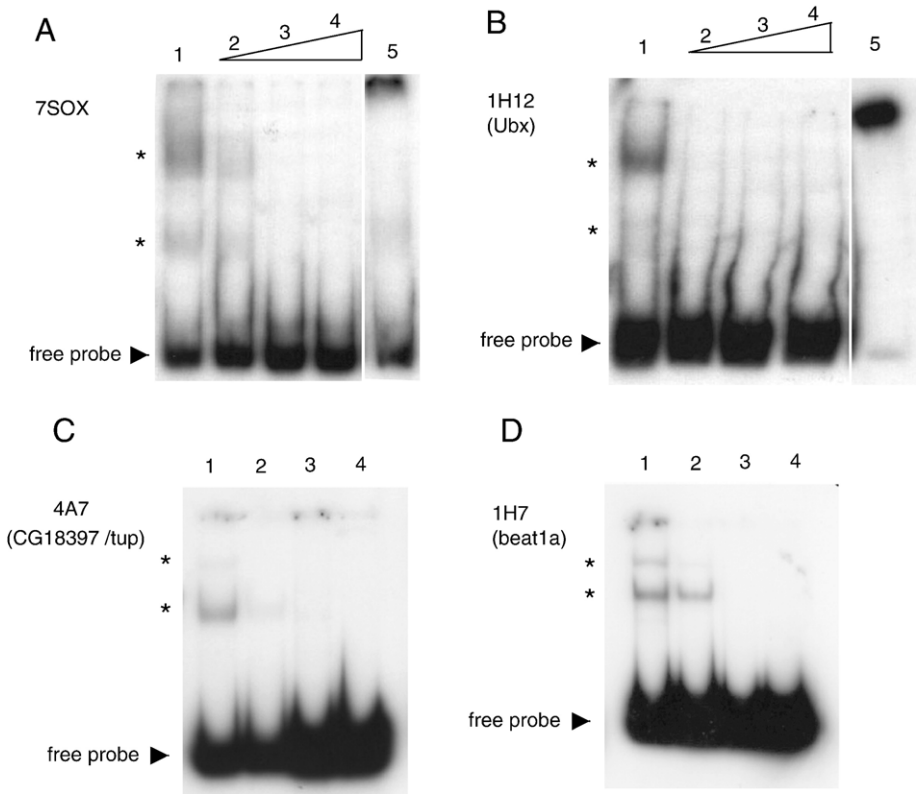

Fig. 2. In vitro binding of SoxN to immunoprecipitated fragments. (A) Binding of SoxN to a probe containing 7 multimerized binding sites for proteins of the Sox family (7SOX probe) (lane 1). This binding is competed with increasing quantities of the same cold probe (lanes 2–4, respectively 10-, 20- and 30-fold molar excess). The complexes are super shifted with SoxN antibody (lane 5). (B) Binding of SoxN to the immunoprecipitated fragment 1H12 (146 bp in length, localized in *Ubx* intron). Specific retarded complexes are observed in the presence of GST-SoxN (lane 1), the formation of which is competed with cold 7SOX probe (lanes 2–4, respectively 10-, 20- and 30-fold molar excess). The complexes are super shifted with SoxN antibody (lane 5). (C–D) Binding of SoxN to the immunoprecipitated fragments 4A7 (localized between *tup* and *CG18397*) and 1H7 (localized in *beat1a* intron). Binding to the ^{32}P labeled DNA probe was tested in the following conditions: incubation with GST-SoxN (lane1) or GST (lane 3), competition of GST-SoxN binding with cold 7SOX probe (20-fold molar excess, lane 2), 4A7 or 1H7 probes alone (lane 4). Note that in the case of 1H7, competition with 7SOX is incomplete, suggesting that the affinity of SoxN for this fragment is higher. In all cases, the retarded DNA/SoxN complexes are indicated with asterisks.

during neuroectoderm differentiation, we analyzed whether both genes were co-expressed later in the VNC. We found that few cells co-expressed Dichaete and SoxN proteins in thoracic segments (3/4 per hemisegment), while no co-localization was observed in abdominal segments (Figs. 3K–M). At stage 16, *eagle* is only expressed in 4 neurons of the 7-3 lineage: three interneurons and one ipsilaterally projecting motor neuron (7-3M, also called GW) (Dittrich et al., 1997). SoxN immunostaining in an *eagle*-Gal4; UAS mCD8-GFP embryo revealed that *SoxN* and *eagle* are co-expressed only in 7-3M/GW motor neuron at stage 16 (Figs. 4A–C). We have not looked at earlier developmental stages, when *eagle* is also expressed in neurons of the NB2-4, NB3-3 and NB6-4 lineages (Dittrich et al., 1997). Since *SoxN* is absolutely required for the formation of lateral *eagle*-expressing NBs (Buescher et al., 2002; Overton et al., 2002), it is possible that more neurons co-express *eagle* and *SoxN* from stages 13 to 14. Double staining experiments with SoxN, even-skipped and engrailed antibodies revealed that SoxN staining was mutually exclusive with both eve (Fig. 4K) and en staining (Fig. 4J). This shows that SoxN is absent from the following *eve*-expressing neurons: aCC, pCC, RP2, CQ and E2, and in the *en* neurons derived from NB1–1, MNB, and row 6 and 7 NBs. Finally, we investigated whether SoxN was co-expressed with one of its potential target gene, *tailup/islet*. By stage 16, *tailup/islet* is expressed in a subset of ventrally projecting motor neurons and interneurons (Thor and Thomas, 1997). SoxN immunostaining in an *islet*-tau-myc-GFP embryo revealed that *SoxN* and *tailup/islet* are co-expressed in each abdominal segment in a pair of neurons on each side of the midline, and in an additional centrally located neuron that might correspond to dopaminergic neuron (Figs. 4D–F). *SoxN* is also co-expressed with two other of its potential target genes, *Ubx* and *Beat1a*. Indeed, while only few *beat1a*-expressing motor neurons are stained with SoxN antibodies (Figs. 6A–B′), several of the *SoxN* expressing cells within the VNC at stage 15/16 also express *Ubx* (Figs. 4G–I).

SoxNeuro involvement in axonal patterning

Based on *SoxN* expression in neurons and glia, and the identification of target genes involved in axon guidance/pathfinding, it is likely that *SoxN* function is also required at later stages of neurogenesis when axon targeting takes place. In the *Drosophila* VNC, most axons are organized in a ladder-like

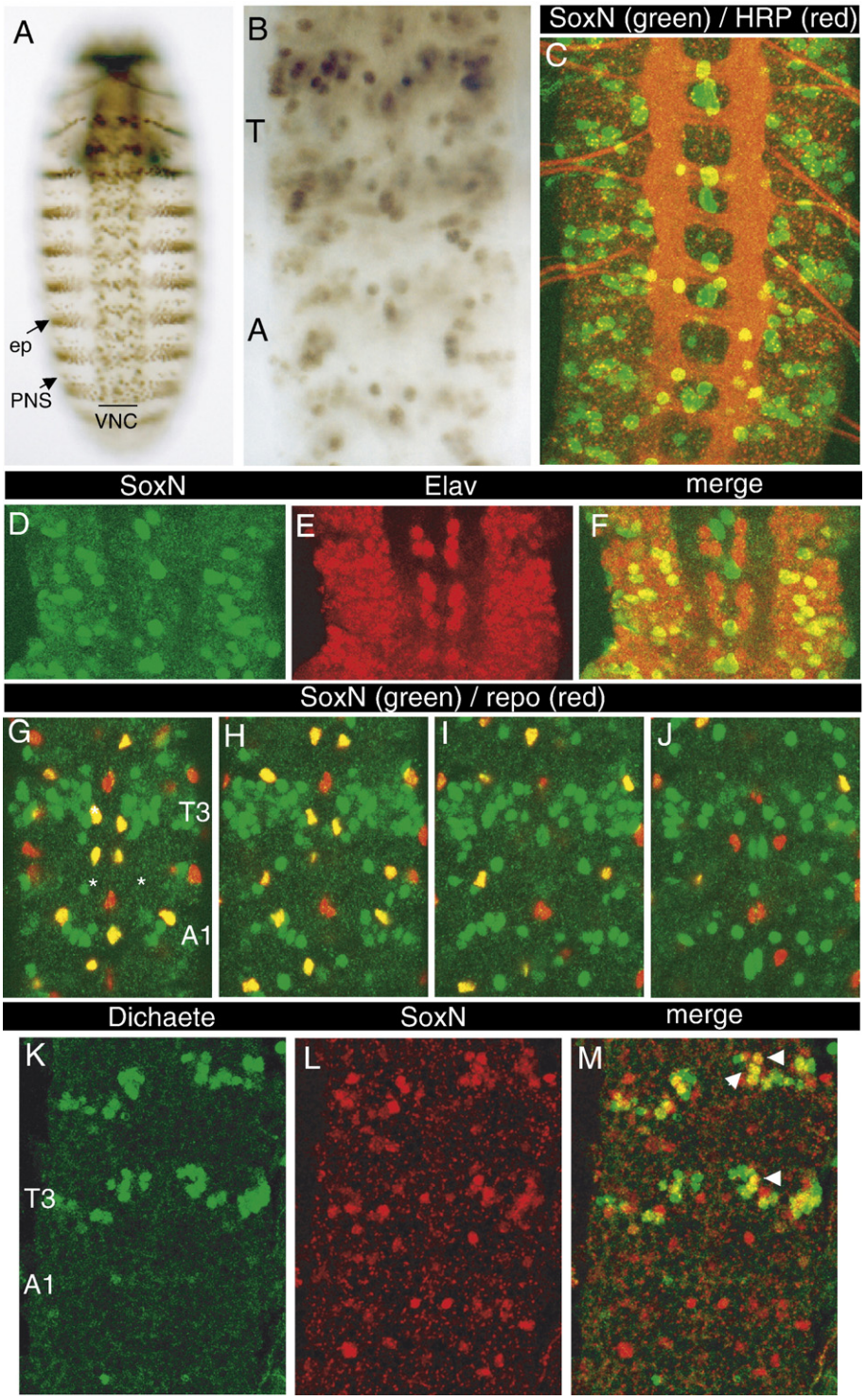

Fig. 3. *SoxN* is expressed in a subset of neurons and glia within the VNC. (A–B) Whole-mount SoxN immunostaining in stage 16 embryos, highlighting *SoxN* expression in the VNC, the PNS and the epidermis (ep). *SoxN* is expressed in a subset of cells in a reiterated segmental pattern in the VNC. Note that staining in the brain is not on the same focal plane. (B) Higher magnification focusing on VNC, showing staining pattern in thoracic (T) and abdominal (A) segments. (C–J) Fillet preparation of wild-type stage 15/16 embryos, immunostained for SoxN and HRP (panel C), SoxN and Elav (panels D–F), SoxN and repo (panels G–J), SoxN and Dichaete (panels K–M). All embryos are oriented anterior up. All images were obtained by confocal microscopy. (D–F) Each panel is a stack of 5 confocal slices (0.8 μM thick) focusing on one segment, showing that SoxN (green in panel D) is expressed in neurons, as revealed with Elav staining (red in panel E). (G–J) Merge images of SoxN (green) and repo (red) staining. All images are stack of 5 confocal slices (0.8 μM thick) focusing on two segments on the same VNC (thoracic T3 and abdominal A1), from ventral-most (G) to dorsal-most (J). SoxN is detected in a limited subset of Repo-positive glia. Note that SoxN is absent from most longitudinal Repo-positive glia (not in the same focal plane). (K–M) Each panel is a stack of 10 confocal slices (0.7 μM thick), focusing on thoracic (T3) and abdominal (A1) segments. SoxN and Dichaete are co-expressed in 3/4 cells per thoracic hemineuromere (marked by arrowhead), while SoxN is absent from the single cell per abdominal hemineuromere expressing Dichaete.

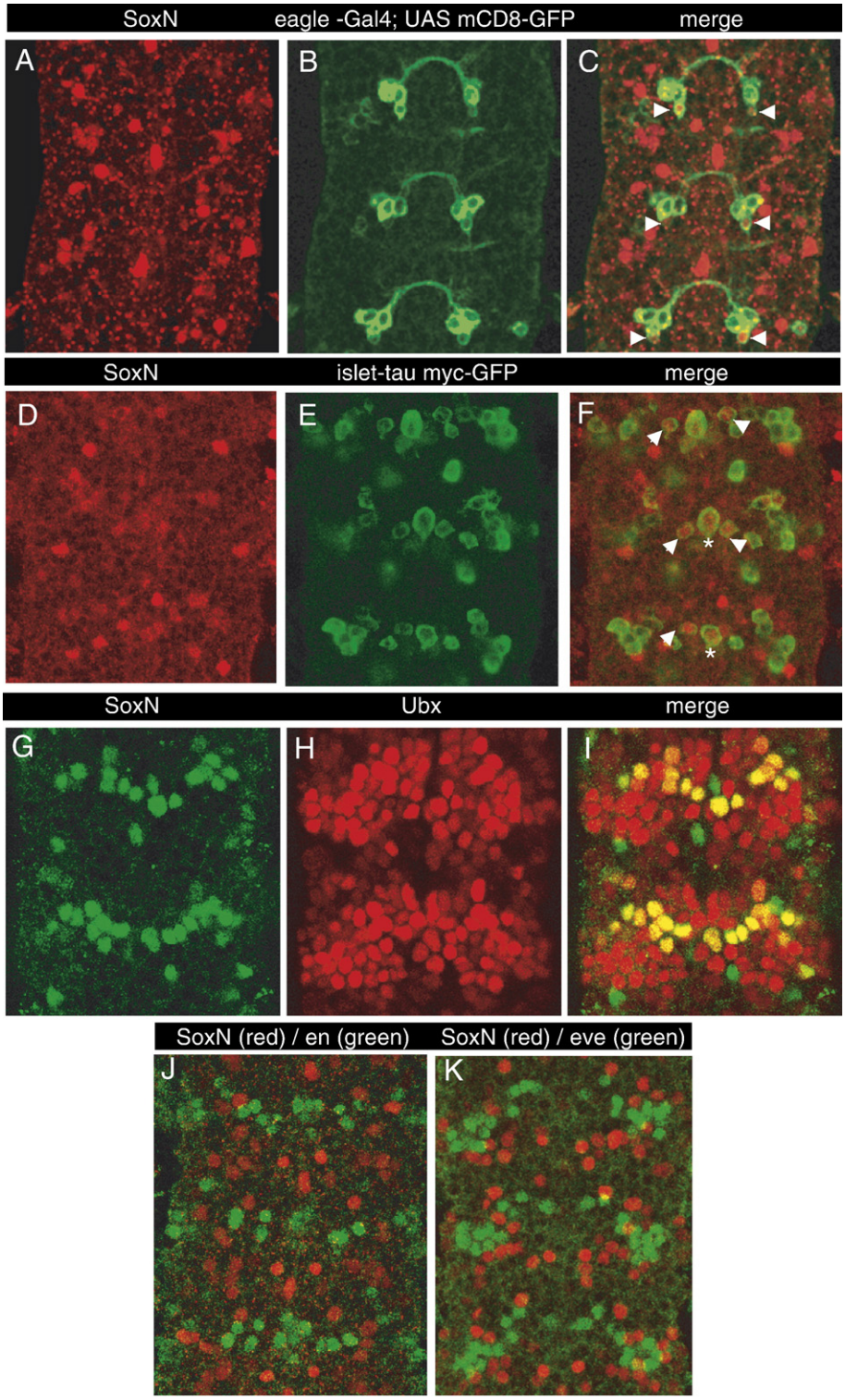

Fig. 4. *SoxN* expression in VNC neurons. SoxN immunostaining was analyzed by confocal microscopy in dissected VNC from embryos at stage 16. All embryos are oriented anterior up. (A–C) Each image is a stack of 5 confocal slices, 0.6 μM thick. SoxN (visualized with anti-SoxN antibody in red, panel A) and *eagle* (*eagle*-Gal4; UAS mCD8-GFP, visualized with anti-GFP in green, panel B) are co-expressed in a pair of GW motor neurons in each segment (marked with arrowheads). (D–F) Each image is a stack of 5 confocal slices, 0.6 μM thick. SoxN (visualized with anti-SoxN in red, panel D) and *islet/tailup* (*islet*-tau-myc-GFP, visualized with anti-myc in green, panel B) are co-expressed in one pair of neurons in each segment (marked with arrowheads), and one cell located at the midline, likely a dopaminergic neuron (marked with asterisks). (G–I) Each image is a stack of 5 confocal slices, 0.8 μM thick. SoxN (visualized with anti-SoxN in green, panel G) and Ubx (visualized with anti-Ubx in red, panel H) are co-expressed in several cells. (J–K) Merge images of SoxN (red) and even-skipped or engrailed (green) immunostainings. Each image is a

pattern, with two segmental commissures across the midline and two longitudinal connectives, connecting individual neuromeres along the antero-posterior axis (Fig. 5A). Embryos lacking *SoxN* show defects in the axon scaffolding in the VNC, including disruption of the longitudinal axon fascicles, and strong defects in the anterior and posterior commissures (Fig. 5B). Since SoxN function is absolutely required in NB formation upstream of the proneural genes, this phenotype could result, at least in part, from the absence of formation of several NBs (Buescher et al., 2002; Overton et al., 2002). Thus, due to its early function in NB formation, it was not possible to analyze *SoxN* late function in neurons by using *SoxN* null mutants, because the defects in CNS scaffolding might be interpreted as a failure of NBs to be formed and to give rise to neurons. Therefore, we decided to use an alternative strategy, consisting in overexpressing a mutated form of SoxN protein interfering with endogenous SoxN function. Indeed, we recently described that SoxN was SUMO modified on Lysine 439, resulting in the repression of its transcriptional activity (Savare et al., 2005). In vivo, overexpression in embryos of a SUMO-deficient and transcriptionally hyperactive SoxN protein (in which the SUMO acceptor Lysine K439 was mutated to Arginine) results in defects in CNS development (Savare et al., 2005). To analyze the function of *SoxN* in neurons, we used the UAS-GAL4 system to drive the expression of this mutated SoxNK439R protein in all neurons using Elav-Gal4. This resulted in specific defects in the axon scaffolding in the VNC, including defects in the commissures (AC and PC are often fused, with higher diameter than in wild type), thickening of the longitudinal connectives, and tangles at the segmental boundaries, suggesting that many axons project inappropriately (Fig. 5C). By contrast, the CNS architecture shows wild-type appearance when overexpressing the wild-type SoxN protein with Elav-Gal4 driver (Fig. 5D), suggesting that the phenotypes rather resulted from perturbation of endogenous *SoxN* function with SoxNK439R in neurons (acting as a dominant negative or hyperactive allele—see below), than simply overexpression of SoxN. This was confirmed by genetic interaction experiments in transheterozygous combinations of both *SoxN* null mutation and mutations in its target genes involved in axonal pathfinding. We reasoned that lowering *SoxN* dose (in a $SoxN^{-/+}$ embryo) should result in a decrease in the expression of its target genes, eventually leading to synthetic lethality and visible axonal scaffolding phenotypes in embryos mutated in only one copy of its target genes. Transheterozygous combinations of *SoxN*/target gene mutations show several degree of synthetic lethality ranging from 100% (*SoxN*/+; *Ubx*/+), 43.2% (*SoxN*/*beat1a*), 19.8% (*SoxN*/*lola*), to 17.8% (*SoxN*/*tup*). By contrast, no lethality was observed in heterozygous combinations of *SoxN*/+, and surprisingly of *SoxN*/*Sema2a* (Table 2). This lethality was correlated with defects in axon scaffolding, such as fusion of commissures, axonal tangles, disruption and/or reduction of longitudinal connectives, as revealed by anti-HRP immunostaining in *SoxN*/*lola* (Fig. 5E) and *SoxN*/*beat1a* embryos (Fig. 5F), while we observed no CNS defect in their sibling single heterozygous (data not shown). For *Ubx*/*SoxN* mutant, no stage 16 embryos were observed, suggesting that lethality period occurred earlier. These results show that *SoxN* and two of its potential target genes, *lola* and *beat1a*, genetically interact in the formation of the CNS axonal architecture.

Finally, we analyzed whether modulating *SoxN* expression could result in perturbation in the expression of one of its downstream target genes, *beat1a*. *Beat1a* expression starts at stage 12 in early born motor neurons, and by stage 14 it is expressed in all motor neurons (Fambrough and Goodman,

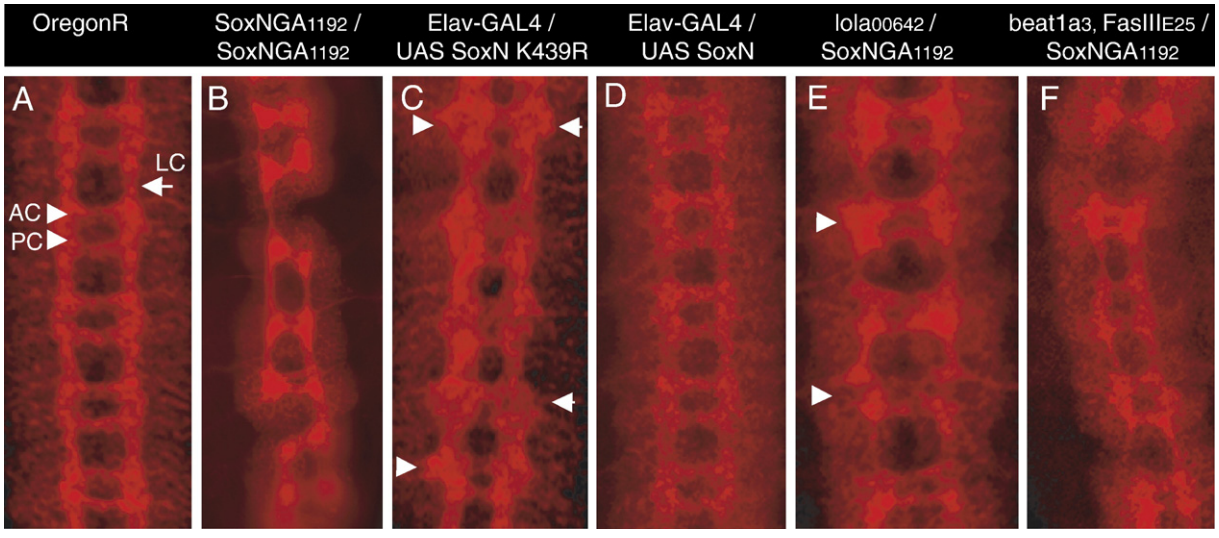

Fig. 5. SoxN involvement in axonal patterning. Cy3 conjugated anti-HRP whole-mount immunostaining performed on stage 16 embryos. The genotypes are indicated. (A) OregonR. Ladder like neuronal structure of the VNC, with anterior commissure (AC), posterior commissure (PC), and longitudinal connectives (LC). (B) In $SoxN^{GA1192}$ homozygous embryos, axonal architecture is severely perturbed, with fusion of the commissures and gaps in LC. C: *elav*-GAL4/+, UAS-SoxNK439R/+. Defects include axonal tangles (arrowheads), thickening of the LC, and partial fusion of commissures. (D) *elav*-GAL4/+, UAS-SoxN/+. The VNC is wild type in appearance. (E) $lola^{00642}/SoxN^{GA1192}$ transheterozygous embryos are characterized by axonal tangles (arrowheads), thinner LC and commissures. (F) In $beat1a^{3}$

Table 2
Analysis of synthetic lethality in transheterozygous mutants

SoxN^{GA1192}/CyO-GFP males crossed to	Lethality of transheterozygous mutants (%)	Number of individuals screened
Females		
+/CyO-GFP	0	222
lola^{00642}/CyO-GFP	19.8	531
beat1a^{3}*FasIII*^{E25}/CyO-GFP	43.2	393
Ubx^{101}/Ser-GFP	100	120
Sema2a^{03021}/CyO-GFP	0	210
Tup^{1}/CyO-GFP	17.8	484

1996). Few of these motor neurons co-express *SoxN* at stages 15/16 (Figs. 6A, B–B′). We found that *beat1a* expression was severely perturbed, although not abolished, in *SoxN* mutant embryos (Fig. 6D). As discussed earlier, this could result from early defects in NB formation, leading to an absence of some of the *beat1a* expressing neurons. As shown before (Fig. 5 and Savare et al., 2005), the hyperactive SUMO-deficient K439R SoxN mutant is able to some extent to interfere with SoxN activity and to induce defects in axonal architecture. We observed that pan-neuronal expression of this mutant form was also able to perturb *beat1a* expression. Indeed, ectopic *beat1a* expression was observed in Elav-GAL4/UAS SoxN K439R embryos (Figs. 6F–F′). In addition, we observed that *beat1a* staining appeared lower than in wild type in several cells (Fig. 6F′). None of these effects were observed when driving the wild-type SoxN form in neurons (Fig. 6G), suggesting that the modulation of *beat1a* expression is likely to result from perturbation of endogenous SoxN activity rather than simply SoxN overexpression. The function of Sox proteins involves partnering with specific co-factors to achieve maximal and specific transcriptional regulation (Kamachi et al., 2000; Wilson and Koopman, 2002; Blanco et al., 2005). The K439R SoxN protein is transcriptionally hyperactive, as shown in cell transfection assays (Savare et al., 2005), and thus could to some extent bypass the requirement of co-factors to interfere with endogenous *beat1a* expression, and other target genes, leading to specific defects in CNS development. As these effects are not observed with overexpression of the endogenous SoxN form, this suggests that the phenotypic effects of SoxNK439R expression do not simply result from SoxN expression, but rather from the ability of SoxNK439R to modulate to some extent endogenous SoxN function (acting for example as a dominant negative or inversely as a hyperactive allele). Collectively, these data support a direct role for SoxN in the regulation of *beat1a* expression,

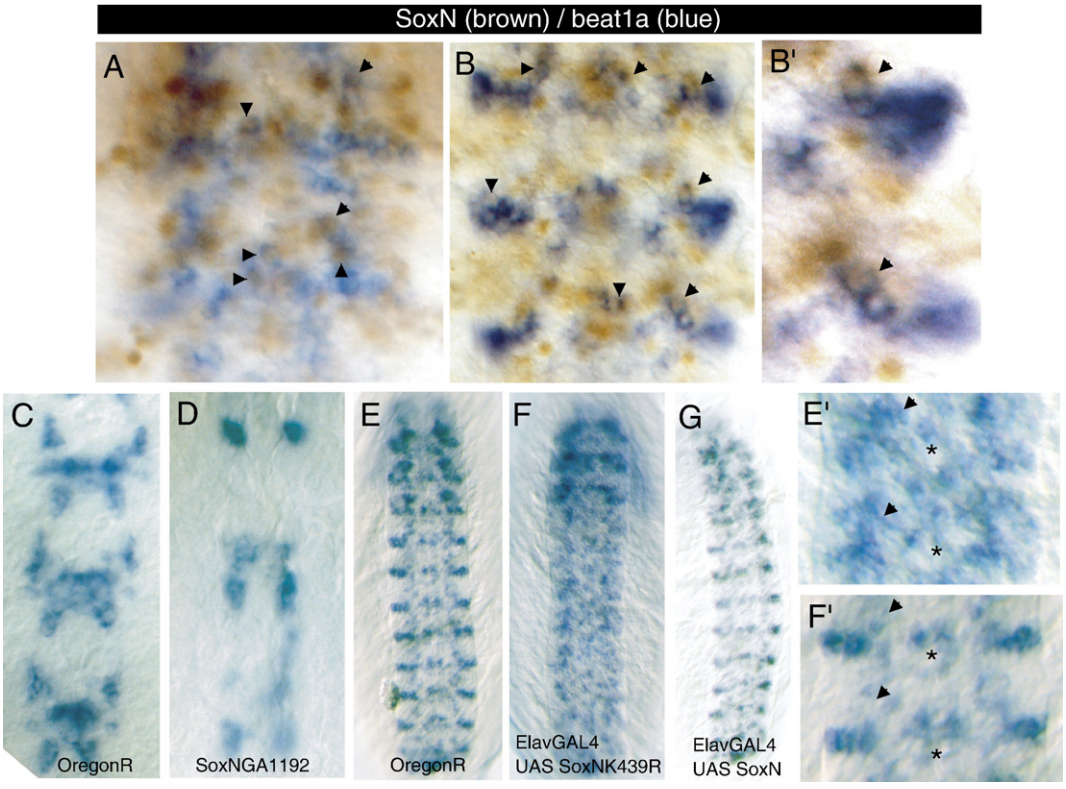

Fig. 6. SoxN regulates *beat1a* expression. (A–B) *beat1a* and *SoxN* are co-expressed in few motor neurons. Whole-mount SoxN immunostaining (nuclear, brown) and *beat1a* in situ hybridization (cytoplasmic, blue) in stage 15 (A) and 16 (B) embryos, focusing on three abdominal segments. Arrowheads point to some of the motor neurons co-expressing *SoxN* and *beat1a*. Panel B′ is a higher magnification of panel B. (C–G) *beat1a* expression is under SoxN regulation, as shown by analyzing *beat1a* expression in *SoxN*-deficient or overexpressing embryos. *beat1a* expression was monitored by in situ hybridization in embryos of the following genotype: (C, E) OregonR, (D) *SoxN*^{GA1192}/*SoxN*^{GA1192}, (F) *elav*-GAL4/UAS SoxN K439R, (G) *elav*-GAL4/UAS SoxN. Images in panels C and D focus on *beat1a* staining in three abdominal segments. Shown are stage 12 (C, D) and stage 16 (E–G) embryos. Panels E′ and F′ are higher magnification of the embryos shown in panels E and F, respectively. All embryos are oriented anterior up.

consistent with the co-expression of *SoxN* and *beat1a* in some motor neurons.

Discussion

We provide here molecular and genetic evidence for a novel role of *SoxN* in axonal patterning, likely through the direct regulation of several genes involved in axon guidance/pathfinding, the identity of which was determined by chromatin immunoprecipitation. We have provided evidence that *SoxN* is expressed in a limited subset of neurons and glia, and is co-expressed in a subset of neurons with at least three of its target genes, *beat1a*, *Ubx* and *tailup*. EMSA experiments showed that SoxN efficiently and specifically binds in vitro to some of the in vivo immunoprecipitated fragments, including those localized in intron (*Ubx*, *beat1a*) and intergenic region (*tup*/*CG18397*). *SoxN* and two of its target genes, *lola* and *beat1a*, genetically interact in the developing CNS, since specific defects in the axonal architecture are observed in transheterozygous embryos. Finally, interfering with the endogenous *SoxN* function through the neuronal specific overexpression of a mutated SUMO-deficient and transcriptionally hyperactive form of SoxN resulted in axon guidance phenotypes. Collectively, these data strongly support a role for the SoxN transcription factor in the regulation of axonal architecture. Intricate relationship between neurons and glia is required during axonal patterning (Edenfeld et al., 2005). As described here, *SoxN* is expressed in subset of both neurons and glia, and could thus potentially contribute to axonal patterning by regulating, positively or negatively, genes expressed by these two cell types. Although the identification of molecules involved in axon guidance/pathfinding has received much attention in the past years, the transcriptional control of this process remains largely unsolved. Several transcription factors expressed in post-mitotic neurons have been shown to regulate axon guidance programs to select specific axon pathways to reach their targets, such as the LIM-homeodomain factors apterous and tailup/islet, which are believed to regulate the expression of guidance cues and their receptors (Shirasaki and Pfaff, 2002; Certel and Thor, 2004). To date, *SoxN* has been involved in dorso-ventral patterning of the neuroectoderm and NB formation in embryonic CNS (Buescher et al., 2002; Overton et al., 2002; Gomez-Skarmeta et al., 2003), and in eye development (Blanco et al., 2005). Our data argue for a novel function of *SoxN* in controlling the formation of CNS architecture through the direct regulation of genes required for axon pathfinding at multiple levels. Indeed, these genes encode proteins acting as pro-adhesive (fas2, DNCad2) and repellent cues (beat1a, Sema2a), and transcription factors expressed in subsets of neurons (tup, Ubx, fru), which in turn can regulate specific axon guidance programs.

Our EMSA experiments showed that the immunoprecipitated DNA are efficiently bound in vitro by SoxN. $^{A}/_{T}^{A}/_{T}$CAA$^{A}/_{T}$ consensus sequences are present in most of these immunoprecipitated fragments (see Supplemental data on line). Further work will be required to determine to which site(s) SoxN binds within these fragments, and whether a more defined consensus emerges. SoxN binds the $^{A}/_{T}^{A}/_{T}$CAA$^{A}/_{T}$ consensus sequence in vitro (this study), and transactivates a reporter gene placed downstream of multimerized consensus sites in cell transfection assays (Bonneaud et al., 2003), as observed for many other Sox. Nevertheless, it has been observed that gene regulatory elements are responsive in vivo to only a small subset of the Sox family, implying that regulatory mechanisms exist to permit selective binding and transcriptional regulation in vivo. Among these mechanisms is the ability of Sox to interact with specific transcription factors, which often bind DNA to adjacent sites, leading to synergistic and context-dependent transcriptional regulation (Kamachi et al., 2000; Wilson and Koopman, 2002). The multiple functions of SoxN are likely to involve differential partnership of SoxN with specific transcription factors. Indeed, it was suggested that SoxN function in the neuroectoderm might involve direct association with the columnar homeodomain transcription factors Vnd/Ind/Msh to regulate *AS-C* gene expression (Buescher et al., 2002; Overton et al., 2002; Zhao and Skeath, 2002; Gomez-Skarmeta et al., 2003). SoxN also associates with the paired type homeodomain transcription factor DPax2 to regulate *crystallin* expression in the cone cells (Blanco et al., 2005). Interestingly, we found in several fragments, including those identified in *lola* and *Sema2a* introns, consensus binding sites for POU-homeodomain transcription factors, which are known to be involved in axon pathfinding (Certel et al., 2000; Certel and Thor, 2004) and to specifically interact with Sox factors in different species (Kamachi et al., 2000). Furthermore, the Sox factor Dichaete was shown to co-regulate with the POU protein Drifter the CNS midline expression of the *Slit* gene, involved in axon guidance at the midline (Ma et al., 2000). Thus, it will be interesting to determine whether the combinatorial activity of SoxN/POU factors contribute to the differential regulation of these genes in the CNS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.08.014.

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